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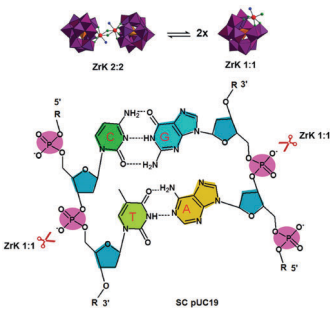

Polyoxometalates as artificial nucleases: hydrolytic cleavage of DNA promoted by a highly negatively charged Zr^{IV}-substituted Keggin polyanion

T. K. N. Luong, I. Govaerts, J. Robben, P. Shestakova and T. N. Parac-Vogt*

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A highly negatively charged binuclear Zr^{IV}-substituted Keggin polyoxometalate $[\{\alpha\text{-}PW_{11}O_{39}Zr(\mu\text{-}OH)(H_2O)\}_2]^{8-}$ (ZrK 2 : 2) has been shown to promote the hydrolytic cleavage of phosphoester bonds in the supercoiled plasmid pUC19DNA under physiological pH and temperature, giving relaxed and linear forms of pUC19 as hydrolysis products. The interaction between ZrK 2 : 2 and DNA was experimentally proven by circular dichroism (CD) spectroscopy and ^{31}P diffusion ordered NMR spectroscopy.

The development of artificial nucleases represents an area of significant interest. However, due to the extreme stability of phosphodiester bonds in DNA, with a half-life for hydrolysis that has been estimated to be 130 000 years under physiological conditions, the development of chemical agents for controlled DNA hydrolysis is a challenging task.[1] Several coordination complexes of Co^{III}, Fe^{III}, Cu^{II} and Zn^{II} were reported to hydrolyze phosphodiester bonds in DNA.[2] Despite the variety in the nature of metal ions and the ligands in the design of artificial metallonucleases, all metal complexes reported so far in the literature carry positive charge, which is assumed to be an essential property in driving the binding to the highly negatively charged DNA backbone.

On the other hand, polyoxometalates (POMs) represent a large class of negatively charged metal–oxygen clusters characterized by a broad diversity of physical and chemical properties. POMs have found applications in materials science,[3,4] medicine[5] and catalysis,[6,7] and have been shown to exhibit a broad range of antiviral, antibacterial and antitumoral properties.

It has been proposed that the high negative charge of POMs plays a key role in their biological activity, mainly by driving electrostatic interactions with various enzymes and proteins.[8–11] However, despite the well-documented biological activity of POMs, there are very few studies that explore their reactivity towards biologically relevant molecules. A few studies have shown that antitumor active POMs can cause cell apoptosis and DNA laddering.[12,13] Although the mechanism of POM induced cell apoptosis remains unclear, the DNA laddering was not directly associated with POM binding to DNA. Our detailed studies with DNA model substrates 4-nitrophenyl phosphate (NPP) and bis-4-nitrophenyl phosphate (BNPP)[14,15] have shown that isopolyoxomolybdates and isopolyoxovanadates were able to promote the hydrolytic cleavage of phosphoester bonds *via* a mechanism that was distinct from that previously established for other artificial metallonucleases. The hydrolytic reaction resulted in the incorporation of the phosphate ion, which was the product of hydrolysis, into the isopolyoxometalate structure, giving catalytically inactive complexes. This so-called "catalyst poisoning" was later avoided by using Zr^{IV}-substituted POMs, which were shown to catalytically hydrolyse phosphoester bonds in small, negatively charged DNA and RNA model phosphoesters NPP and BNPP, while retaining their structure.[16–19] Although the interaction between POMs and DNA seems counterintuitive, as both molecules carry a high negative charge, intrigued by the results involving successful hydrolysis of DNA model systems, in this study we explore the phosphodiesterase activity of highly negatively charged Zr^{IV}-substituted polyoxometalate $[\{\alpha\text{-}PW_{11}O_{39}Zr(\mu\text{-}OH)(H_2O)\}]^{8-}$(ZrK 2 : 2), towards the hydrolysis of supercoiled plasmid pUC19 DNA (SC pUC19), a circular double-stranded DNA containing 2686 base pairs.

Depending on conditions, in aqueous solutions ZrK 2 : 2 undergoes multiple equilibria and can convert into $[Zr(PW_{11}O_{39})_2]^{10-}$(ZrK 1 : 2)[18] or $[\alpha\text{-}PW_{11}O_{39}Zr(OH)(H_2O)]^{4-}$(ZrK 1 : 1) species (Fig. 1).[19] Due to the lack of free coordination sites ZrK 1 : 2 was assumed to be catalytically inactive, while ZrK 1 : 1 is expected to exhibit a higher catalytic activity compared to ZrK 2 : 2.[16] However, to the best of our knowledge, the synthesis and isolation of pure ZrK 1 : 1 under neutral pH conditions has not been reported, precluding its direct application for DNA cleavage in Tris-HCl buffer at pH 7.0.[16,20] The speciation behavior of ZrK 2 : 2 in D_2O was previously studied;[16,18] however, as the hydrolytic experiments with DNA required the use of Tris-HCl buffer, its speciation in the presence of this buffer has been examined. The results (Fig. S1–S8, ESI†) show that the presence of

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† Electronic supplementary information (ESI) available: ^{31}P and ^{31}P DOSY NMR spectra, electrophoresis gel at 37 °C, CD spectrum, ln[SC pUC19] as a function of time, and kinetic data on the influence of temperature. See DOI: 10.1039/c6cc08555e

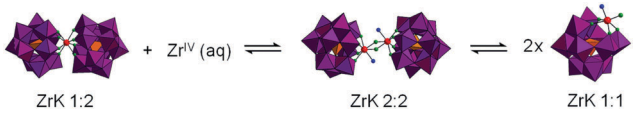
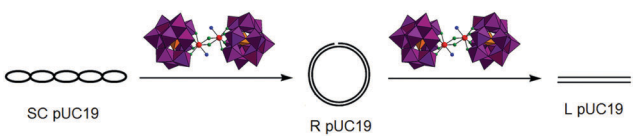


Fig. 1 Structures and equilibrium of ZrK 2:2, ZrK 1:2 and ZrK 1:1. The WO_6 groups are represented by violet octahedra, while the internal PO_4 groups are represented by orange tetrahedra. Zr^{IV}, H_2O molecules and OH groups are represented by big red, blue and green balls, respectively.

buffer does not significantly affect the speciation equilibrium, and that the conversion of ZrK 2:2 into inactive ZrK 1:2 is favoured by increasing pH, the initial concentration of ZrK 2:2, temperature, incubation time and ionic strength. The results showed that in 10 mM Tris-HCl buffer ZrK 2:2 is stable at pH 7.0 for at least up to 7 days at 50 °C, and therefore these conditions were chosen for the hydrolytic studies with pUC19.

The hydrolysis of supercoiled plasmid pUC19 DNA (SC pUC19) in the presence of ZrK 2:2 (Scheme 1) is conveniently followed by agarose gel electrophoresis. In general, SC pUC19 can be hydrolysed initially in two consecutive steps. In the first step, a single cut or nick in a strand of SC pUC19 results in the relaxed circular form (R pUC19). In the next step, another break of the complementary strand within approximately 16 base pairs of the initial cut site leads to the linear form (L pUC19).[21,22]

Fig. 2 shows an example of the agarose gel electrophoresis of pUC19 hydrolysis in the presence of ZrK 2:2 in Tris-HCl buffer at pH 7.0 and 50 °C at different time intervals. Lane 2 is untreated pUC19 obtained after purification from producing cells and is used as a reference. It includes 81% of monomeric supercoiled (lower band) and 19% of multimeric forms (unresolved upper band). Lane 3 is HindIII-cleaved pUC19 corresponding to the linear monomeric plasmid (L pUC19) of 2886 bp. During the course of pUC19 hydrolysis, the monomeric supercoiled form in the mixture converts into a closed-circular or relaxed form (R pUC19) by a nicking reaction and its band is expected to be below the band of the multimeric forms. The double-strand cleavage of SC and catenated pUC19 results in L pUC19. The phosphodiester bond hydrolysis is evidenced by the gradual disappearance of the SC pUC19 band, and the appearance of R pUC19 and subsequently of L pUC19. A similar hydrolysis pattern was also observed at 37 °C (Fig. S9, ESI†).



Scheme 1 Hydrolysis of pUC19 in the presence of ZrK 2:2.

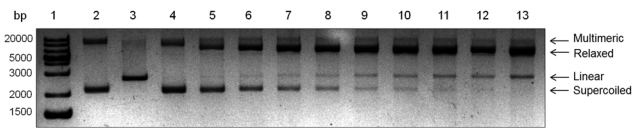


Fig. 2 Electrophoresis gel of the hydrolysis of 20 ng μL^{-1} plasmid pUC19 DNA in the presence of 0.25 mM ZrK 2:2 in 10 mM Tris-HCl at pH 7.0 and 50 °C. Lane 1: 1 kb plus DNA ladder, lane 2: pUC19 alone after sample preparation and without heating, lane 3: linear pUC19 alone, lane 4: after mixing, lane 5 to lane 13: after 8 h, 17 h, 23 h, 26 h, 32 h, 35 h, 39.5 h, 43 h and 46 h incubation, respectively.

Based on the integration values of the bands of different forms of pUC19, the rate constant of SC pUC19 hydrolysis was calculated by measuring the percentage of different pUC19 forms as a function of time. The percentages of supercoiled, relaxed and linear forms of pUC19 at different time intervals are shown in Fig. S10 (ESI†). During the course of the hydrolytic reaction, SC disappeared completely after 46 hours while the percentages of R and L increased to 87% and 13.5%, respectively. The natural logarithm of the SC concentration as a function of time (Fig. S11, ESI†) was fitted to a first-order linear decay function, resulting in a rate constant of $30.50(\pm 2.20) \times 10^{-6}$ s^{-1}. The incubation of the reaction mixture for longer period resulted in a faint DNA smear that was observed under the linear band, while no smear was present in the control sample. This indicates that ZrK 2:2 continues to hydrolyse the linearized pUC19, albeit slowly. Cleavage appears to occur randomly, as sequence specificity would have resulted in the formation of distinct shorter bands.

Several control experiments were performed to ensure that ZrK 2:2 was indeed responsible for phosphodiester bond hydrolysis in DNA. At 50 °C, the reaction between SC pUC19 and the monolacunary Keggin POM ($[\alpha\text{-}PW_{11}O_{39}]^{7-}$) showed no evidence of SC cleavage, indicating that the embedded Zr^{IV} ions are responsible for the observed reactivity. Under similar conditions, SC pUC19 hydrolysis promoted by ZrK 1:2 was about 5 times slower ($k_{obs} = 7.13 (\pm 0.44) \times 10^{-6}$ s^{-1}) in comparison to ZrK 2:2. The reaction between the Zr^{4+} salt ($ZrCl_2O{\cdot}8H_2O$) and SC pUC19 was also examined; however, due to the formation of insoluble Zr^{IV} hydroxyl polymeric gels,[23] no band in the agarose gel could be observed.[19] The absence of gels in the reaction mixture indicates that the equilibrium shift resulting into free Zr^{4+} was not very significant under DNA hydrolysis conditions.

The influence of temperature on the hydrolytic reaction rate constants was determined on a solution containing 20 ng μL^{-1} pUC19 and 0.25 mM ZrK 2:2 in 10 mM Tris-HCl at pH 7.0 at different temperatures ranging from 37 °C to 55 °C. From the data plot shown in Fig. S12A (ESI†), the activation energy (147.47 ± 0.21 kJ mol^{-1}) was calculated from the Arrhenius equation, which is lower than that in the absence of ZrK 2:2 under identical reaction conditions (170.43 ± 2.93 kJ mol^{-1}). Based on the Eyring equation (Fig. S12B, ESI†), the enthalpy of activation ($\Delta H^{\ddagger}$ = 144.80 ± 2.21 kJ mol^{-1}) and the entropy of activation ($\Delta S^{\ddagger}$ = 117.35 ± 1.29 J mol^{-1} K^{-1}) were calculated, resulting in the Gibbs activation energy ($\Delta G^{\ddagger}$) of 108.43 ± 1.73 kJ mol^{-1} at 37 °C, which is very similar to $\Delta G^{\ddagger}$ of BNPP hydrolysis (111.12 kJ mol^{-1} at 37 °C) promoted by ZrK 2:2.[18] The positive value of $\Delta S^{\ddagger}$ suggests that the interaction between DNA and Zr^{IV} in ZrK 2:2 is likely to replace counter cations from the compact inner layer or the defuse outer layer surrounding DNA. This counter ion release process is nearly entirely entropic resulting in positive $\Delta S^{\ddagger}$ values.[24]

The binding between ZrK 2:2 and DNA is the critical step for DNA hydrolysis.[25] The binding of Keggin type POMs to calf thymus DNA (ctDNA) has been previously examined in the context of their anti-tumoral activity. The data ruled out the intercalating binding mode and proposed the groove or outside stacking binding of these POMs with ctDNA.[26] Circular dichroism (CD) spectroscopy is a

useful tool for studying the binding process, as bands originating from the base stacking (275 nm) or the right-handed helicity (247 nm) are sensitive to the mode of DNA interactions with small molecules.[24,25,27–31] The ctDNA was also used in hydrolysis experiments with ZrK 2 : 2; however, as ctDNA is a complex mixture of linear DNA fragments of different lengths, fragmentation products are difficult to characterize, and single-strand cuts remain unnoticed. When ZrK 2 : 2 was added into the ctDNA solution, the intensities of both bands increased (Fig. S13, ESI†), indicating that the interaction between ZrK 2 : 2 and ctDNA took place. The intercalation mode of binding could be excluded because this type of interaction would lead to intensity decrease of both bands.[28,30]

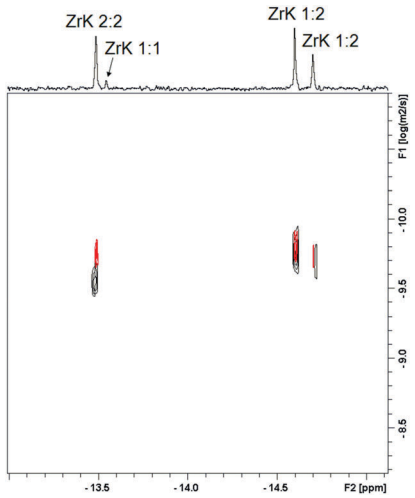


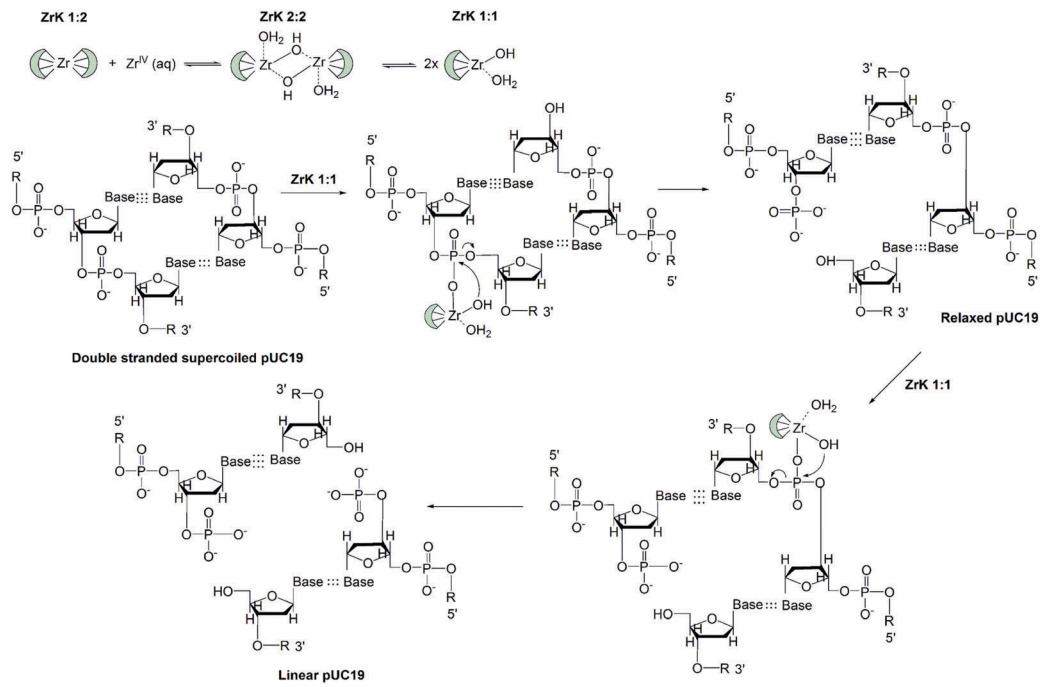
Fig. 3 ^{31}P DOSY spectra of 3.0 mM ZrK 2 : 2 (black) and of a mixture of 3.0 mM ZrK 2 : 2 and 0.32 mM ctDNA in D_2O at pH 7.0 and 293 K (red).

The increase of both bands in the CD spectra suggested that the possible binding mode of ZrK 2 : 2 to DNA is rather electrostatic in nature and most likely occurs between the positive charge of Zr^{4+} and the negative charge of the phosphate group of the DNA helix.[24,30] The increase in CD intensity of both bands actually indicates that this interaction stabilizes the structure of DNA.[24,30]

The interaction between phosphodiester bonds in DNA and ZrK 2 : 2 was further observed by measuring ^{31}P NMR of 3.0 mM ZrK 2 : 2 in the absence and in the presence of ctDNA at pH 7.0 (Fig. S14, ESI†). The addition of ctDNA to a solution of ZrK 2 : 2 resulted in the appearance of a ZrK 1 : 1 signal at −13.70 ppm, indicating that ctDNA could promote the conversion of ZrK 2 : 2 into ZrK 1 : 1.[18,19] This observation is analogous to our previous finding that interaction with proteins facilitates the conversion of dimeric Zr^{IV}-POMs into the monomeric form.[32]

^{31}P diffusion ordered NMR spectroscopy (DOSY) was further applied in order to obtain evidence for the interaction between ctDNA and ZrK 2 : 2. However, the equilibria between ZrK 2 : 2, ZrK 1 : 2 and ZrK 1 : 1 complicate the straightforward use of this technique as observed diffusion coefficients represent a population-weighted average of the species involved in the equilibrium.[16] Therefore, ^{31}P DOSY spectra were recorded at two different temperatures (298 K and 280 K) in the absence and presence of ctDNA and the results are shown in Fig. 3 and Table S1 (ESI†). In the absence of ctDNA at 298 K, two POM species with different diffusion coefficients were observed, which correspond to ZrK 2 : 2 and ZrK 1 : 2. Due to the low intensity of ZrK 1 : 1, its DOSY signal could not be observed. In the presence of ctDNA (Fig. 3, red), the diffusion coefficient of ZrK 2 : 2 is significantly lowered, indicating interaction with DNA.

In the absence of ctDNA at 280 K (Fig. S15, ESI†), the difference in the diffusion coefficients between ZrK 2 : 2 and ZrK 1 : 2 is smaller than at 298 K (1.08×10^{-10} m^2 s^{-1}), indicating, as expected, a slower



Scheme 2 Proposed reaction mechanism for the hydrolysis of pUC19 DNA promoted by ZrK 2 : 2.

conversion of ZrK 2:2 into ZrK 1:1 at lower temperatures. Interestingly, the addition of ctDNA did not result in significant changes in the ^{31}P DOSY spectra, indicating that the interaction between Zr-POMs and ctDNA is not pronounced at this temperature.

Based on the speciation of ZrK 2:2, kinetics and the binding studies described above, a mechanism of SC pUC19 hydrolysis in the presence of ZrK 2:2 was proposed, as shown in Scheme 2.[16,18,19,33] Previous DFT calculations on small phosphoester model systems suggested that ZrK 1:1 is more catalytically active as compared to ZrK 1:2 and ZrK 2:2, since its Zr^{IV} ion has several exposed coordination sites that can be replaced by the substrate or that can act as a nucleophile.[16] It is therefore reasonable to assume that ZrK 1:1, which has been identified by ^{31}P DOSY NMR spectroscopy in this study, is also the main species reacting with DNA. Although counterintuitive, the interaction between negatively charged ZrK 1:1 POM and DNA can be compared to association of anions in water, which is a well-documented phenomenon.[18,34,35] It can be also related to association between two negatively charged POM monomers to form a dimeric POM, also known as "anion–anion" dimerization, which is proposed to occur through interactions with H_3O^+ or *via* counterions that screen the charges of the negatively charged species to reduce their mutual repulsions.[36,37] In the proposed mechanism, in the first step ZrK 1:1 coordinates to the oxygen atom of a phosphate group of one single strand of SC pUC19, resulting in a more positive charge at the P atom, thus activating it toward a nucleophilic attack. The OH group or the coordinated water of ZrK 1:1 can play the role of a nucleophile, attacking the phosphorous atom and leading to the cleavage of the P–O bond and forming R pUC19.[16] In the second step, ZrK 1:1 binds to the oxygen atom of a phosphate group of the complementary strand, activating the phosphodiester bond toward hydrolysis in a similar manner as described above. According to a previous literature, the cleavage in the second strand occurs within approximately 16 base pairs of the initial cut site, as evidenced by the appearance of linear form L pUC19 in the electrophoresis gels.[21,22]

In conclusion, we report on the first example of hydrolytic cleavage of DNA promoted by a highly negatively charged polyoxometalate complex under physiological pH conditions. The interaction between POM and DNA has been evidenced by several complementary techniques, and is proposed to occur between Zr^{IV} and the oxygen atom of the phosphodiester bond, which has been previously evidenced in DNA model systems.[16,18,38] The interaction between negatively charged DNA and monomeric Zr-POM is likely to occur *via* a phenomenon similar to the association of anions in water. The current study demonstrates the potential of Zr^{IV}-substituted POMs as artificial nucleases and contributes to the further development of POMs as Lewis acid catalysts for the hydrolysis of biomolecules. It may also shed more light on the molecular origin of biological properties of metal-substituted POMs, as it indicates that despite their high negative charge they are able to interact with DNA and cause hydrolysis of phosphodiester bonds under physiologically relevant conditions.

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